Development of *Colletotrichum acutatum* on tolerant and susceptible *Olea europaea* L. cultivars: a microscopic analysis

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Abstract

Colletotrichum acutatum is a cosmopolitan and damaging plant pathogen of temperate, subtropical and tropical fruits, and causes anthracnose on olive (*Olea europaea* L.). Three olive cultivars showing a variable response to infection by *C. acutatum* were selected to a preliminary study of pathogen development. Fruit samples, from susceptible and tolerant cultivars, were taken at 0, 24, 48, 72 and 192 hours after inoculation for a microscopic and histological study of the infection and colonization process. The aim of this study was to compare the infection process: conidial germination, germ tube and appressorium formation, hyphal growth and mesocarp colonization in susceptible and tolerant olive cultivars as a condition for further exploration of disease development, which is required to develop cultivars with improved resistance to anthracnose. The rate of mesocarp colonization differed among the susceptible and tolerant cultivars, and both intracellular hemibiotrophy and subcuticular intramural necrotrophy were observed. Hemibiotrophic infection predominated in the moderately-tolerant cultivar.

Introduction

The genus *Colletotrichum* includes many of the most damaging plant pathogens, such as *C. acutatum*, *fragariae* and *gloeosporioides* [1]. *C. acutatum* is the causal agent of anthracnose and blight on cereals, grasses, legumes, vegetables, perennial crops and a number of tree fruit [2-3]. It can infect all plant surfaces, but favours young leaves, branches and fruits of herbaceous species growing in a humid climate [3]. A clear understanding of the olive-pathogen interactions is essential because when olive (*Olea europaea* L.) fruit are infected, the quality, stability and flavour of the oil can all be compromised [4-5]. Although some olive cultivars are known to be highly tolerant and others susceptible, the genetic basis of this variation is nowadays unknown. Current management strategies rely on the use of tolerant cultivars and the use of cultural, chemical and biological controls [3]. The enhancement of tolerance remains a major objective for many breeding programmes, especially in lower-yielding regions and developing countries [1, 6]. The critical step for a further infection is the interaction between cuticle and pathogen. The cuticle is the first site of contact of fungus in the environments, and therefore is thought to play a significant role in fruit-fungus interactions. The initial stages of olive infection are very similar with other hosts *Colletotrichum* interaction. The host-pathogen interaction starts with (a) conidial adhesion to the host surfaces, (b) conidia germination, (c) appressorium development and differentiation to form penetration pegs that enter through the host cuticle, (d) growth and colonization on the host tissues.

Two types of infection strategy - intracellular hemibiotrophic and subcuticular intramural necrotrophic life style - or a combination of these are commonly used by *Colletotrichum* spp. to gain access to the host cells [2, 7-9]. In hemibiotrophic infections, a symptomless biotrophic phase is followed by a destructive necrotrophic one, during which symptoms become apparent. Examples of pathogens which employ this strategy are *C. graminicola* [10], *gloeosporioides* [11], and *lindemuthianum* [12-13]. In the subcuticular intramural infection strategy, rather than penetrating the epidermal cell wall, the fungus grows under the cuticle and within the periclinal and anticlinal walls of epidermal cells [9, 3, 14]. *C. phomoides* [2], *capsici* [15] and *circinans* [2] behave in this manner. *Colletotrichum* spp. infection strategies have been investigated in a range of plant species [14, 16-20]. However, the infection process by *C. acutatum* in tolerant and susceptible olive cultivars is not well described at this time, particularly at the ultrastructural level. The objective of this work was to follow the infection process, colonization, structures (conidial germination, germ tube and appressorium formation) and strategies adopted by *C. acutatum* on three olive cultivars, which vary in terms of tolerance to the pathogen, using fluorescent microscopy.

Materials and Methods

Plant and fungal material

The three olive cultivars 'Galega' (susceptible, highly infected with high yield lost due to fruit drop and complete destruction of olive orchards after consecutive attacks), 'Cobrançosa' (moderately-tolerant, inhibits fungus development in the fruit during the first hours after inoculation. The pathogen may reduce the tree growth and olive fruit production with some fruit drop) and 'Picual' (tolerant, inhibits fungus development in the fruit and does not affect trees vigour) were used, based on their known differential response to *C. acutatum* infection. The fruit were obtained from an olive orchard at the National Station of Plant Breeding in Elvas, Portugal.

Inoculation

The pathogen was isolated from diseased olive fruit of cultivar 'Picual', collected from the Alentejo region of Portugal. The fungus was cultured on potato dextrose agar (PDA) for 8 days at 22ºC under a 12 hour photoperiod. Inoculum was prepared by flooding dishes with sterile distilled water, scraping the surface gently with a glass rod, and filtering the resulting suspension through sterile cheesecloth. The inoculum comprised a suspension adjusted to 10^5 - 10^6 spores ml^{-1} of sterile water and was used to infect the three olive cultivars. Inoculation experiments consisted of three replicates of 3 olive trees per cultivar. In three consecutive years, ten olive fruits were collected and analysed.

Sample fixation

In order to study *C. acutatum* colonization of the olive cultivars, all branches were vaporized with a spore suspension and enclosed in plastic bags after inoculation, to create a high humidity conditions in order to encourage conidial germination and development. Control plants were sprayed with water and the branches bagged in the same way. Fruit were sampled at 0, 24, 48, 72 and 192 hours after inoculation (hai), and symptom appearance was recorded on a daily basis. For microscopic analysis, the fruits were snap-frozen in liquid nitrogen and stored at -80ºC.

Microscopic assessment

Fruits were evaluated for conidial germination, germ tube and appressorium formation, host membrane invasion, hyphal growth and mesocarp colonization. The fruit were sectioned under distilled water using a Vibratome Series 1000plus (TAAB Laboratories Equipment Ltd., Aldermarston, UK). The ~20 µm thick sections, containing 2-3 cell layers, were mounted on multi-well slides (ICN Biomedicals Inc., Costa Mesa, CA, USA), pre-treated as described by Prieto et al. [21], and analysed by fluorescence microscopy using an Axioskop2 MOT microscope fitted with an AxioCam HRC

camera (Carl Zeiss MicroImaging Inc.). Samples were analysed by Carl Zeiss Laser Scanning System LSM5 PASCAL software (Carl Zeiss, Jena GmbH).

Results

Under favourable environmental conditions, conidial germination began at 48 hai; 90% of the conidia germinated by 48 hai (Figs.1a and 1b). No lesions were observed on olive fruit epidermis, in all cultivars, at 24 hai (not shown). Section concerning lenticels revealed that no conidia germination was observed at 24 hai. In culture, the conidia were ellipsoid (Fig. 1b). During the germination process, a septum on the conidium was visible (Fig. 1c). Germinating conidia produced either long or short germ tubes, with or without appressoria (Fig. 1d). Differentiation to new conidia was occasionally visible (Fig. 1d). Single conidial germ tubes were produced at the spore tip and by 72 hai, most of the germ tubes had differentiated into appressoria. Appressoria were formed at the tip of the germ tube (Fig. 1c). In some mature appressoria, an internal light spot and a dark pigment was observed under fluorescence microscopy at 72 hai (Fig. 1b). When the appressoria began to darken, the host penetration process began. During the necrotrophic phase, a large mass of mycelium developed in the inner spaces of the mesocarp. Based on the microscopic observations, the development pattern of conidia, germ tubes and appressoria was identical on all three cultivars.

Typical anthracnose symptoms were observed when the fruit surface became wrinkled (Fig. 2a), but the most characteristic symptom was the formation of circular dark, sunken lesions containing orange conidial masses on the surface immature and ripe fruit (Fig. 2b). Within 72-192 hai, the lesion diameter reached ~10 mm (Fig. 2c).

The differences in susceptible and tolerant cultivars were expressed after pathogen penetration, which in olive cultivars, occurred exclusively via infection pegs. Temporal differences, related to pathogen expansion and proliferation inside fruit were observed when the mesocarp, was observed by fluorescence microscopy. During the first hours after inoculation the pathogen was not observed in the mesocarp of cultivars studied. The mesocarp of the three cultivars was clear without any signs of infection. At 48 hai the differences became evident among susceptible (´Galega'), moderately-tolerant (´Cobrançosa') and tolerant ('Picual') cultivars. At this time 'Galega' and 'Cobrançosa' cultivars showed clear signs of infection, such as, hyphae and mycelium development in the mesocarp (Figs. 3a, 3b, respectively). After 48 hai necrotic lesions and dark necrotic spots were observed in 'Galega' throughout all the fruit, from the mesocarp to the endocarp (Fig. 3a). However, no infection structures were visible at this time in the tolerant cultivar 'Picual' (Fig. 3c). In this way, the major differences between susceptible and tolerant cultivars were related to the major or minor capacity of pathogen to colonized olive mesocarp. Based on the fluorescence microscopic observations a tolerance scale was developed, such that cultivar 'Picual' was the most tolerant and cultivar 'Galega' the most susceptible of the three cultivars under study (Figs. 3d, 3e, 3f). Hyphae invaded the mesocarp of 'Galega' and

‘Cobrançosa’ within 48 hai, while in ‘Picual’, the first sign of infection did not become visible until 72 hai (Fig. 3f). Once the pathogen had invaded the host cells, the mesocarp became densely colonized; causing host tissue dehydration, and finally the appearance of necrotic and dark brown lesions (Fig. 3d). Following this stage, the mesocarp became completely colonized by secondary hyphae, that grew both inter- and intracellularly, leading to a total collapse of the host cells (Fig. 3d).

In the mesocarp of whole fruit and sectioned of them, both hemibiotrophic and the subcuticular intramural modes of infection were observed. As shown in figure 4a, penetration pegs develop into a narrow infection vesicle hypha that ramify throughout ‘Cobrançosa’ mesocarp inter- and intracellularly, which is characteristic of intracellular colonization. In ´Galega’ and ‘Picual’ cultivars the pathogen developed across walls of epidermal cells (Figs. 4b, 4c, respectively). The fungus did not immediately penetrate the cell lumen; instead it grew within periclinal and anticlinal host walls characteristic of subcuticular intramural necrotrophic infection (Fig. 4b). At the end of both types of infection, host cells became gradually colonized by narrow secondary hyphae (Fig. 4c) which radiated from multilobed vesicles that rapidly colonized surrounding host cells culminating with rupture of the cuticle. In all microscopic observations, even after 192 hai, no acervuli erupted through the cuticle in all cultivars studied.

Discussion

The morphology of the *C. acutatum* conidia (shape, size and septation), appressoria, and the infection process among olive cultivars studied were similar to those observed in other *Colletotrichum* pathosystems, such as, *C. acutatum* / strawberry [14] and / almond interactions [3].

Conidial germination was observed only at 48 hai, mainly in the cultivar ‘Galega’. When 24 hai fruit sections were observed no conidia were detected on the cuticles’. Conidia structures were more frequently observed on the cuticle of ‘Galega’ than on the other two cultivars studied, consequently, appressoria development was observed more frequently in this susceptible cultivar. Kubo [22] stated that appressoria promotes pathogen adhesion to the host surface and provides the mechanical force and enzymes needed for initial penetration. Lower disease incidence and less severity can be related to lower appressoria numbers [19]. According to our microscopic observations, appressorium development is a prerequisite of invasion olive fruit by *C. acutatum.*

Our experiments have shown that after a symptomless period of a few hours, the fungus spreads throughout the host mesocarp, killing the host cells and dissolving cell walls ahead of infection (Fig. 3d). The infection of *C. acutatum* on olive fruit is slower than observed on almond leaves [23] (3 hai) or strawberry tissues [14] (4 hai). The same is true for *C. truncatum* on pea pods [24] (4 hai). However, the time of infection increases when *C. dematium* and *C. lindemuthianum* infects cowpea stems (6 to 12 hai, respectively [25-26]). In citrus [27], almond [23], strawberry [28],

and blueberry [29] germination and germ-tube differentiation occurs within a few hours under favourable environmental conditions. In olive fruit, this infection seems to be delayed to pos 24 hai, this is probably due to the cuticle composition, which has high levels of lipids that constitutes a barrier for conidia adhesion. Another possibility is related to the amount of water on fruit surface that can influence the interaction among pathogen and olive fruit [41].

A septation was observed in mature conidia, just as has been seen in *C. lindemuthianum* on cowpea [26], *C. graminicola* on four turfgrass species [30], *C. destructivum* on alfalfa [31] and *C. acutatum* on strawberry and almond [14, 19].

A unique germ tube, with different lengths formed directly above conidia cells, with no particular orientation (Figs. 1c, 1d). The same pattern was observed in the *C. destructivum*-tobacco interaction [16]. However, germ tube development doesn't seem to be a prerequisite for appressoria or internal light spot formation. Once, mature conidia, with a germ tube developed, appressoria with an internal light spot on the germ tube ends (Figs. 1b, 1c). The role of the internal light spot (*ILS*) within the melanized appressorium is unknown. Its presence has been related to pore formation and consequent by host penetration [23], or to peg penetration and the subsequent formation of an infection structure [27]. Leandro et al. [28] found that there was no direct correlation between the *ILS* structure and the infection process.

As was reported for other pathosystems [15] the pathogen enters olive fruit directly though fruit cuticle other types of host penetration such as penetration through stomata [2] was not observed in the pathosystem under study.

This preliminary study suggests that both hemibiotrophic and subcuticular intramural necrotrophic, were present. However, no correlation between olive cultivars susceptibility/tolerance and the infection pathways adopted from pathogen was found. Both intracellular hemibiotrophy and subcuticular intramural necrotrophy are exploited by the pathogen [8], as occurs also in the interaction between *C. acutatum* and citrus, blueberry and almond. Wharton and Schilder [20] related infection and colonization strategies with the susceptibility of the host tissue colonized. In the present study, we have shown that the pathogen used subcuticular intramural necrotrophy as an infection strategy for both susceptible and tolerant olive cultivars (Figs. 4b, 4c). However, only in moderately-tolerant cultivar 'Cobrançosa' both colonization strategies were observed (Fig. 4a).

Severity of infection was greater on 'Galega' tissues than on any of the other cultivars included in this study. In 'Galega', necrotic lesions were observed not only at the fungus infection site but they also spread to numerous cells, during the first hours of infection, while in 'Cobrançosa' and 'Picual' a few small necrotic lesions were restricted to the area under the cuticle, becoming visible between 48 and 72 hai, respectively. No eruption of acervuli through the olive cuticle was observed up to 192 hai, as was also found after 168 hai in the *C. graminicola / Poa annua* and / *P. pratensis* pathosystems [30]. Different studies reported the appearance of acervuli in other *Colletotrichum* spp. 70 hai in *C. dematium* [25] and 120 hai in *C. destructivum* [32]. The lack of symptom expressed by 'Picual' during the early period

of infection is an indication of the presence of tolerance rather than resistance factors. Several types of resistance response has been related to the induced cellular lignification and programmed cell death, phytoalexin biosynthesis, secretion of pathogenesis-related proteins, fruit sugar content and phenolic compounds [33, 34, 35, 36]. A preliminary biochemical study on 'Galega' and 'Cobrançosa' fruits revealed that the differences found among the total phenolic compounds cannot be related to the different levels of tolerance found within these cultivars (unpublished data). However, this study considers the fruits at a particular stage of ripening. Nevertheless, 'Galega' is a cultivar that ripens very early, end of September – beginning of October. At this time its total phenolic compounds have decreased. This can be the reason why this cultivar is severely attacked by *C. acutatum*, which finds ideal conditions for germination and colonization at this period (first rainfall). Both 'Cobrançosa' and 'Picual' cultivars ripen latter, normally by the end of November. When *C. acututum* attacks they still have very high levels of phenolic compounds, preventing an higher colonization of this pathogen. High levels of phenolic compounds have been related to the tolerance of onion to *C. circinans* [37]. Further determination of individual biochemical compounds should be considered in order to relate them to olive fruit tolerance, once that they are highly dependent on the olive genotype [38].

Bentes and Matsuoka [39], studying the *C. guaranicola* infection process on resistant and susceptible clones from *Paullinia cupana* var. *Sorbilis*, observed temporal differences in tissue colonization. In the field, differences in olive tissue colonization, between tolerant and susceptible cultivars have been reported [40]. However, a microscopic study to analyse these temporal differences and to characterise all structures involved in olive attack has never been reported.

To date there has been no detailed ultrastructure-based description of the infection process of *C. acutatum* in olive fruits. This study provides a platform for a deeper exploration of the *C. acutatum* / olive interaction, giving indications of possible mechanisms to overcome this devastating disease. In what concerns 'Galega' an early harvesting can avoid the infection by the disease preventing both olive orchard destruction and pathogen dissemination. Studies need to proceed in this area that is still unexplored and is of major importance for olive growers.

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Legends

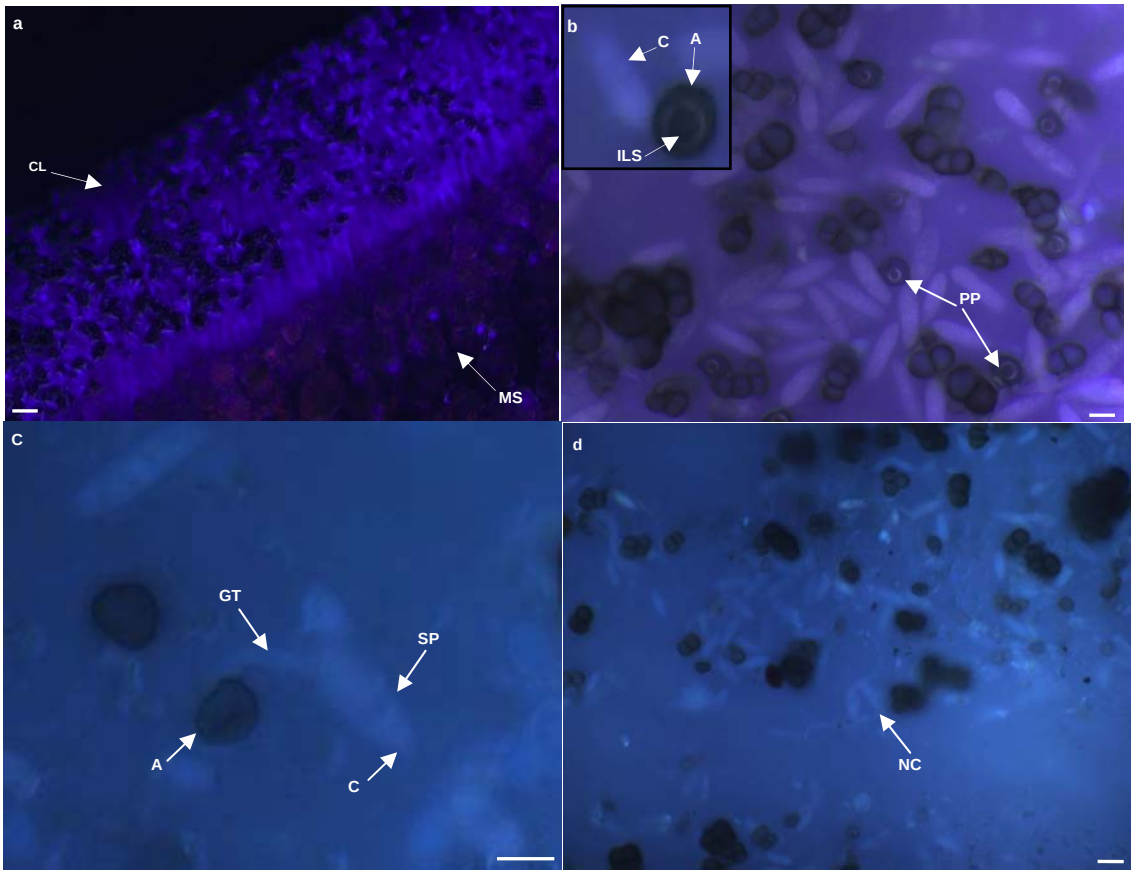

Fig. 1 *C. acutatum* infection structures in olive fruit. **a**. Massive adhesion of fusiform conidia on the cultivar 'Galega' cuticle was observed at 48 hai. **b**. Germinated and ungerminated conidia on the fruit surface 48 hai; a penetration pore has differentiated under a mature appressorium and internal light spot (*ILS*) were observed at 72 hai. **c**. Conidia (with a septum at the equatorial zone (arrow)) develop a germ tube at the end of which a pigmented fluorescing appressorium differentiated within 72 hai. **d.** The development of one or more secondary conidia simultaneously was observed at 48 hai. Scale bar represents 20 µm in panel a, 10 µm in panels b and d, and 5 µm in panel c. CL= host cuticle; MS= mesocarp; C= conidium; A= appressorium; ILS= internal light spot; PP= penetration pore; GT= germ tubes; SP= septum; NC= new conidial formation.

Fig. 2 Developmental stages of *C. acutatum* infection on 'Galega' fruit. **a**. and **b**. Typical lesions first appear as a small depression in the cuticle 192 hai, which later turns dark brown. **c**. After 192 hai, the infection has developed dark fruiting bodies covering the fruit surface, from which white to orange conidial masses emerge.

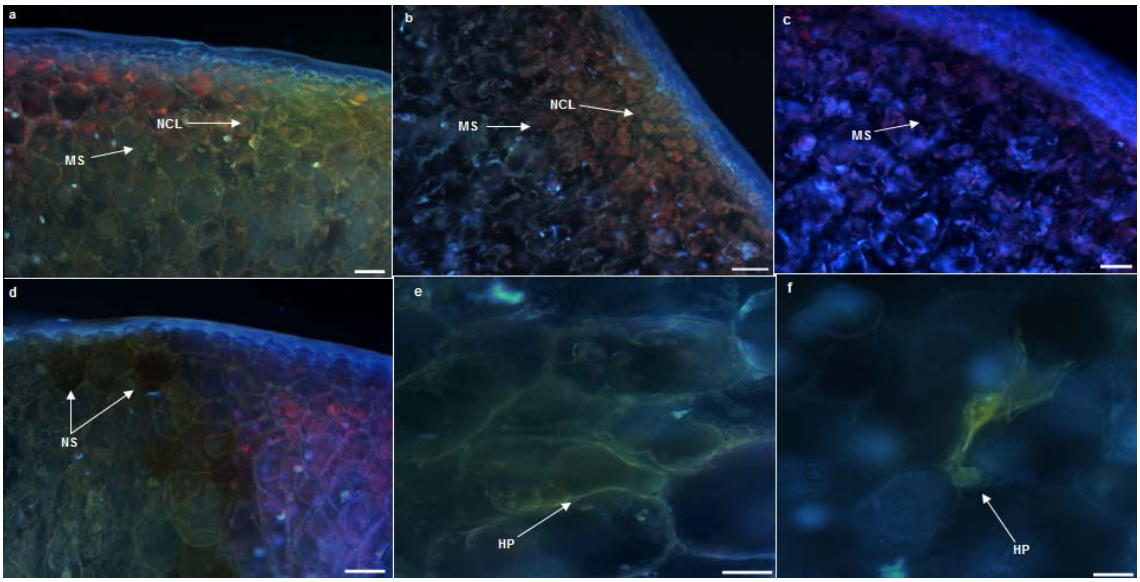

Fig. 3 a. Mesocarp of susceptible cultivar 'Galega'at 48 hai, **b**. Moderately-tolerant cultivar 'Cobrançosa' at 48 hai, and **c**. Tolerant cultivar 'Picual' at 48 hai. **a**. At 48 hai 'Galega' mesocarp showed signs of infection. Fungal growth becomes visible between cellular spaces and the cellular disorganization of mesocarp is a consequence of fungus colonization. In the susceptible cultivar, cells beneath cuticle were completely necrotic. **b**. In cultivar 'Cobrançosa' the first small evidence of fungal growth was observed at 48 hai and was restricted to a small necrosis under cuticle (arrow). **c.** Healthy cuticle, exocarp and mesocarp tissues were observed in tolerant cultivar 'Picual'. **d-f**. Temporal differences on pathogen host colonization in susceptible (d), moderately-tolerant (e) and tolerant (f) cultivars were observed at 72 hai. **d**. In the susceptible cultivar, large necrotic lesions with necrotic spots were observed for all tissues, culminating with cellular collapse at 72 hai. **e.** In the moderately-tolerant cultivar some necrotic cells were observed. Thick hyphae spread from the exocarp to endocarp of 'Cobrançosa' 72 hai, before starting an extended necrotrophic phase involving all fruit tissue. **f**. In the tolerant cultivar, 'Picual', no infection was observed in the mesocarp while other restricted cells

small symptoms were visible at 72 hai. Scale bar represents 20 µm in panels a, b and c; 10 µm in panel d; 5 µm in panels e and f. NCL= necrotic cells; MS= mesocarp; NS= necrotic spots; HP= hyphae.

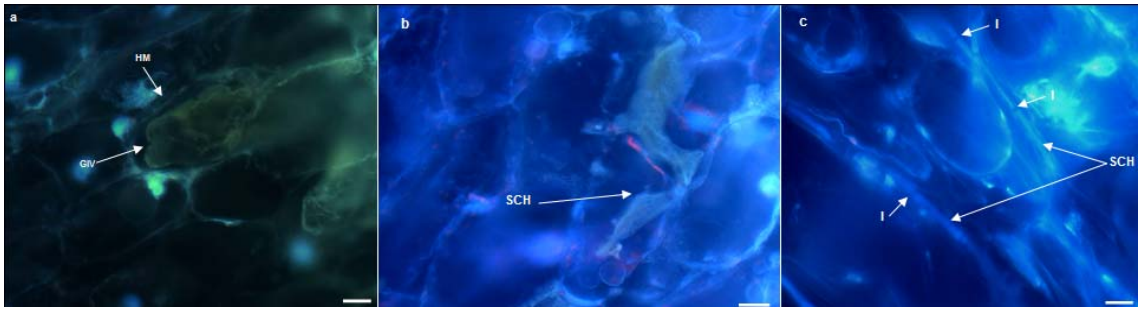

Fig. 4 a. Intracellular hemibiotrophic-like infection structures in infected ‘Cobrançosa’ fruit mesocarp cells. Penetration of epidermal cells and fungal development inside the host were observed. The host membrane (arrow) encloses a globose infection vesicle without damage the host plasma membrane. **b-c**. Subcuticular infection vesicles were observed in ‘Galega’ (b) and ‘Picual’ (c) fruit. Penetration of a hypha was observed in ‘Galega’ but no disturbance of the cell lumen was observed. During the first stage of infection, hyphae grow inside the host cell wall of ‘Galega’ without penetrating the lumen. **c**. Development of hyphae with internodes is marked with arrows. Scale bar represents 10 µm in all panels. HM= host membrane; GIV= globose infection vesicle; I= internodes; SCH= subcuticular hypha.